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STRUCTURES OF MACROMOLECULAR COMPOUNDS

X-ray Diffraction Study of Highly Purified Human Ceruloplasmin

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Abstract—The three-dimensional structure of ceruloplasmin (CP) with unoccupied labile metal-binding sites and the structure of CP containing Ni^{2+} in the labile sites were solved for the first time at 2.6 and 2.95 Å resolution, respectively. Crystallization was performed with the use of storage-stable CP, which was prepared in the presence of proteinase inhibitors and purified from (pre)proteinases. Ceruloplasmin with Ni^{2+} crystallized in the orthorhombic space group, which had been earlier unknown for CP. Ceruloplasmin with the unoccupied labile sites crystallized in the trigonal crystal form. The differences in intermolecular contacts observed in the trigonal and orthorhombic crystal structures of CP are considered. The conformational changes attendant upon Ni^{2+} binding are described. It was suggested that the labile sites are multifunctional and can both bind metal ions potentially toxic to organisms and be involved in electron transfer from substrates to the active site.

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INTRODUCTION

Ceruloplasmin (CP, ferro-O_2-oxidoreductase, EC 1.16.3.1) is a multifunctional copper-containing oxidase. This protein was discovered in 1944 in vertebrate plasma [1]. In humans it accounts for 95% of plasma copper. The physiological meaning of ferroxidase activity of CP was elucidated. Ceruloplasmin is implicated in iron metabolism by catalyzing oxidation of Fe^{2+} and thus facilitating the incorporation of Fe^{3+} into apo transferrins [2]. Ceruloplasmin oxidizes four Fe^{2+} ions and is involved in four-electron transfer to oxygen giving rise to water, thus hindering nonenzymatic oxidation of iron producing free radicals. The inherited defect of the CP gene (aceruloplasminemia) is a systemic disorder of iron metabolism and degeneration of the central nervous system, the pancreas, and other tissues [3]. In addition, CP exhibits superoxide dismutase activity [4] and glutathione-dependent peroxidase activity [5] and serves as the physiological inhibitor of the prooxidant leukocyte enzyme myeloperoxidase [6].

The first CP crystals were grown as early as 1960 [7]. In 1996 the three-dimensional structure of CP was solved at 3.1 Å resolution. Ceruloplasmin is a monomer composed of six domains with six tightly bound copper ions [8]. Domains 2, 4, and 6 contain homologous mononuclear copper-binding sites. The trinuclear site is located between domains 1 and 6. In addition, there are two labile metal-binding sites, half of which in native CP are occupied [9]. Investigations of the binding of Cu^{2+}, Co^{2+}, Fe^{2+}, and Fe^{3+} ions in the labile sites of CP suggested that these sites play an important role in iron oxidation [9]. The binding sites of the inhibitor (azide ion) and various substrates (biogenic and synthetic amines) were revealed [10]. Recently, the structure of CP was solved at 2.8 Å resolution [11]. This made it possible to reveal the Ca^{2+}- and Na^{+}-binding sites for the first time.

There are several factors that make it difficult to grow CP crystals of high diffraction quality. Ceruloplasmin is a large multidomain molecule with a weight of 132 kDa and a carbohydrate content of 7–8%. This protein is very sensitive to proteolytic degradation. The isolation of undegraded and stable CP has remained a difficult problem since its discovery [12–15]. Ceruloplasmin samples contain impurities of prothrombin [16] and unidentified metalloproteinases [17], owing to which it is necessary to use proteinase inhibitors in all steps of CP isolation [18]. In the course of isolation, CP is prone to aggregation, which also hinders the crystallization.

We isolated CP by the method described earlier [19]. This method allows the preparation of homogeneous storage-stable undegraded CP. According to this method, proteinase inhibitors (EDTA and phenylmethylsulfonyl fluoride) were added to blood plasma. Impurities of unidentified proteinases and prothrombin were

adsorbed with the use of arginine- and heparin-Sepharose. Since the presence of metalloproteinase in CP samples was reported earlier [17], we estimated the stability of CP after the addition of Ni^{2+} to blood plasma.

In the present study, we describe the characteristic features of the CP structure with the unoccupied labile sites (2.6 Å resolution, the trigonal crystal form) and the structure of the CP complex with Ni^{2+} ions (2.95 Å resolution, the orthorhombic crystal form). The differences in the intermolecular contacts observed in the trigonal and orthorhombic crystal structures of CP are analyzed. The specific binding of Ca^{2+} ions to CP is discussed. The conformational changes attendant upon Ni^{2+} binding in the labile sites were revealed. Hypotheses concerning the role of the labile metal-binding sites of CP are suggested.

MATERIALS AND METHODS

Isolation of CP and analysis of samples. Ceruloplasmin was isolated from blood plasma by affinity chromatography on protamine-Sepharose [19]. Blood plasma of healthy people (3 l) was diluted twofold with 100 mM sodium acetate buffer, pH 5.5, with the addition of EDTA and phenylmethylsulfonyl fluoride to the final concentrations of 1 and 0.1 mM, respectively. This plasma was applied to a DEAE-Sephadex A-50 column (10 × 5 cm) equilibrated with 50 mM sodium acetate buffer, pH 5.5; washed with the same buffer until $A_{280} < 0.005$ in the flow-through solution; and eluted with a linear NaCl gradient (100-ml portions, 0 ⟶ 0.4 M NaCl in 50 mM sodium acetate buffer, pH 5.5). Blue fractions were combined and cooled on ice. An equal volume of an ethanol–chloroform mixture (9 : 1, *v*/*v*) was added to these fractions. After 20 min, the mixture was centrifuged at 6000*g* for 15 min at 4°C. The supernatant was withdrawn, and an equal volume of an ethanol–chloroform mixture (9 : 1, *v*/*v*) was added. After 20 min, the mixture was centrifuged under the same conditions. The blue CP-containing precipitate was dissolved in PBS (0.15 M NaCl, 10 mM sodium phosphate buffer, pH 7.4) and centrifuged at 15000*g* for 15 min at 4°C to remove undissolved impurities. Then CP was filtered through a PBS-equilibrated arginine-Sepharose column (10 × 2.5 cm). Ceruloplasmin was eluted with PBS in the free volume of the column. Then CP was applied to a PBS-equilibrated protamine-Sepharose column (10 × 2.5 cm). The column was washed until $A_{280} < 0.003$ in the flow-through solution and eluted with 0.3 M NaCl in 30 mM *tris*-HCl buffer, pH 7.4, at the maximum rate. The CP-containing fractions were combined, diluted threefold with water, and filtered through a PBS-equilibrated heparin-Sepharose column (4 × 1 cm). Ceruloplasmin was eluted with PBS in the free volume.

Another CP sample was isolated according to the same scheme from blood plasma, to which $NiCl_2$ was added (instead of EDTA) to the final concentration of 1 μM. The CP samples were concentrated with the use of a Vivaspin 20 cell to the final concentration of 80 mg/ml. The homogeneity of CP was verified by PAG electrophoresis in the absence of detergents [20]. Ceruloplasmin was specifically stained in the gel with *o*-dianisidine [21], which allowed us to reveal possible oligomeric forms. The degree of proteolytic degradation of CP was estimated by Ds-Na-PAG electrophoresis [22]. The concentration of homogeneous CP was determined by spectrophotometry using the coefficients A_{280} = 1.61 ml/mg per centimeter and A_{610} = 0.0741 ml/mg per centimeter [15]. The A_{610}/A_{280} ratio related to the ratio of type-I copper ions to aromatic amino-acid residues in CP was used as the criterion of the quality of purified CP. The atomic absorption analysis was carried out on an AAS-5000 PerkinElmer instrument.

Crystallization and X-ray data collection. Crystals of CP complexes were grown by the vapor-diffusion method at 4°C. The crystallization solution analogous to that used earlier [21] contained 3–7% PEG 20 000 with an addition of 1–2% PEG 1000, 20 mM $CaCl_2$, 200 mM NaCl, and 50 mM sodium acetate buffer, pH 5.5. Blue crystals with a size of 0.08–0.2 mm appeared within 5–7 days; 30% glycerol or 28% PEG 400 were used as the cryoprotector. The binding of calcium and nickel in the crystals was confirmed by X-ray fluorescence. X-ray diffraction data were collected on the beamline BW6 at DESY (Hamburg) at 100 K using a MAR CCD detector. Anomalous-scattering data were collected near the Ni-absorption edge to precisely determine the Ni^{2+}-binding sites. The optimal data-collection parameters were chosen with the use of the BEST program [23]. The data were processed using the DENZO and SCALEPACK programs [24]. The X-ray data-collection statistics are given in Table 1.

Structure solution and refinement. The structures were solved by the molecular replacement method with the use of the MOLREP program [26] starting from the atomic coordinates of CP, whose structure had been solved earlier at 3.1 Å resolution [8]. The refinement was carried out with the use of the REFMAC program and the COOT graphical program [26, 27]. The final R and R_{free} factors for CP containing no copper ions in the labile sites were 21.4 and 26.5%, respectively. For the CP structure with Ni^{2+}, the final R factor (R_{free} factor) was 18.6% (25.1%). The Ramachandran plot shows that 86.6 and 87.1% of the φ and ψ torsion angles in the amino-acid residues are in the most-favored regions, 12.8 and 12.2% are in the additionally allowed regions, 0.5 and 0.6% are in the generously allowed regions, and 0.1 and 0.1% are in the disallowed regions for CP containing no copper ions in the labile sites and the CP complex, respectively. Characteristics of the models are given in Table 2.

Table 1. X-ray data-collection statistics

	CP-Ca^{2+}	CP-Ca^{2+} + Ni^{2+}	CP-Ca^{2+} + Ni^{2+}
Wavelength, Å*	0.97	0.97	1.48
Resolution range, Å	30–2.6	30–2.95	30–3.25
Last resolution shell, Å	2.64–2.6	3.0–2.95	3.31–3.25
Sp. gr.	$P3_221$	$I222$	$I222$
Unit-cell parameters, Å	$a = b = 210.78$ $c = 84.5$	$a = 74.84$ $b = 226.66$ $c = 233.7$	$a = 74.84$ $b = 226.66$ $c = 233.7$
Number of independent reflections	64294 (3104)*	34841 (1729)	60352 (3018)
Completeness, %	95.9 (93.4)	82.3 (83.2)	99.7 (100)
$I/\sigma(I)$	17.7 (1.75)	10.6 (2.64)	8.9 (3.6)
R_{merge}	4.5 (30.5)	5.7 (23.5)	8.7 (35.6)

* The values for the last resolution shell are given in parentheses.

RESULTS AND DISCUSSION

Characterization of CP. Human CP isolated in the present study is characterized by the ratio A_{610}/A_{280} = 0.052, which is larger than that determined for CP in the earlier studies on crystallization (0.048) [8]. The analysis of CP by Ds-Na-PAG electrophoresis showed that 95% of the protein exists in the intact state with *M* of around ~132 kDa (Fig. 1a). The PAG electrophoresis in the absence of detergents revealed no oligomeric forms of CP (Figs. 1b and 1c). The electrophoretic properties of CP isolated from blood plasma in the presence of $NiCl_2$ are analogous to those of the former sample. The addition of Ni^{2+} ions to the starting blood plasma did not lead to an increase in the degree of degradation of CP in the course of isolation, which could result from the activation of metalloproteinase. The crystals grown from this CP were found to contain Ni^{2+}. The atomic absorption analysis showed that both CP samples contain 5.95 ± 0.05 mol of copper per mole of the protein. No proteolytic degradation of CP was observed by Ds-Na-PAG electrophoresis after storage of CP in the presence of the bacteriostatic agent (sodium merthiolate) for two months at 37°C.

General structural organization of CP. Ceruloplasmin is a pseudohexamer consisting of six domains related by a noncrystallographic threefold axis. The structural organization of each domain is typical of cupredoxins. The domains contain a β barrel with a long loop between the first and last β sheets, which covers the top of the barrel (Fig. 2). The presence of these long flexible loops, which do not form strong bonds with the compact core, on the molecular surface is, apparently, responsible for high sensitivity of CP to proteolytic degradation. The limited proteolysis with trypsin affords the same fragments; i.e., the cleavage always proceeds at the particular peptide bonds located after R481, R701, and K887 [13, 15]. The replacement of these amino-acid residues by site-directed mutagenesis produced human CP resistant to proteolysis [28]. The residues R481, R701, and K887 are conserved proteolysis sites, because the loops covering the tops of the β barrels in CP from other animals contain residues homologous to those mentioned above. In addition, some other peptide bonds in these loops are also susceptible to proteolytic attack, for example, by elastase [29].

The structure of human CP was solved at the presently highest resolution (2.6 Å). The reliability and accuracy of the structural information obtained by X-ray diffraction depend primarily on the spatial resolution of the diffraction experiment. In the case of CP, an improvement of the resolution allowed us to reveal the positions of particular amino-acid residues, which had not been included in the earlier model. The orientation of the residue His980, which serves as a ligand for the copper ion in the trinuclear center and plays an important role in the catalytic properties, was determined with higher accuracy (see Fig. 3). The same orientation of this residue was observed in the CP structure solved at 2.8 Å resolution [11]. In the earlier model, the residue His980 is twisted by about ~87° [8].

Table 2. Characteristics of the refined structures of the ceruloplasmin complexes

	CP-Ca^{2+}	CP-Ca^{2+} + Ni^{2+}
Resolution, Å	20–2.60	20–2.95
Number of non-hydrogen atoms	8376	8376
R/R_{free} factor, %	21.4/26.5	18.6/25.1
rms deviations from ideal values:		
for interatomic distances	0.020	0.020
for bond angles, deg	1.45	1.48
Temperature factor, $Å^2$	47.8	48.4

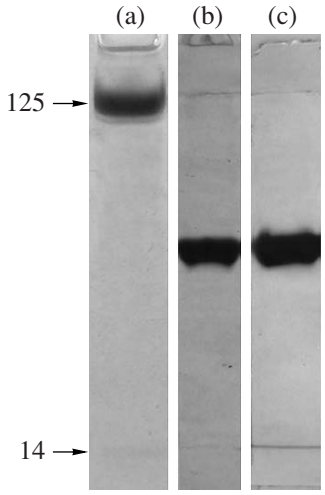

Fig. 1. Electrophoretic analysis of human ceruloplasmin (20 μg per lane). (a) Ds-Na-PAG electrophoresis, Coomassie R-250 staining, the arrows on the left indicating the molecular-weight markers (in kDa); (b, c) PAG electrophoresis in the absence of detergents: (b) Coomassie R-250 staining, (c) *o*-dianisidine staining.

In spite of the higher resolution compared to that for the structures solved earlier [8, 11] and the use of undegraded CP, we failed to determine the positions of the amino-acid residues involved in the interdomain loops 339–346, 475–482, 885–891, and 1041–1046. At the same time, the region 700–709 containing the proteolysis site R701 was reliably determined in all known CP structures. The factors responsible for this situation are considered below.

We found three Na^+ ions and one Ca^{2+} ion bound to CP. This is analogous to the structure solved earlier at 2.8 Å resolution [11]. The sodium-binding sites are located between domains 1 and 3, domains 3 and 4, and domains 5 and 6. The Ca^{2+} ion is located in domain 1 and is coordinated by D127 and D128 as bidentate ligands, the main-chain oxygen atoms of the residues K109 and Q124, and two water molecules. The average distance to the ligands is 2.42 Å.

In spite of the fact that, according to biochemical data, three to four Ca^{2+} ions can be bound to the CP molecule [30], no other binding sites were found for this metal in CP. It is known that the presence of Ca^{2+} ions in solution influences the binding of CP to AE-agarose [30]. It is commonly accepted that the selective affinity of CP for chelate sorbents (in particular, for AE-agarose) is attributed to the presence of clusters of negatively charged amino acids on the surface of the CP molecule [31]. Actually, we found the following evolutionally conserved clusters arranged as a broad area on the surface of human CP: 242–255, 586–595, 605–616, 740–752, 924–933, and 940–955 [19]. In our experiments the synthetic polyanionic peptides corresponding to the D–Q–V–D–K–E–D–E–D–F–Q–E (586–597), E–V–E–W–D–Y–S–P–Q–R–E–W–E (740–752), and D–E–N–E–S–W–Y–L–D–D (924–933) regions elute CP from AE-agarose [32], which confirms the importance of these regions for the binding to chelate sorbents. The influence of Ca^{2+} ions on the binding of CP to AE-agarose is, apparently, associated with the ion-exchange effect rather than with specific binding, as was suggested earlier [30]. The only specific Ca^{2+}-binding site was located in domain 1 of the molecule remote from the above-mentioned negatively charged clusters.

Crystal packing of CP. Ceruloplasmin crystallized in two different space groups. The trigonal crystal structure was obtained earlier [8, 11]. In the present study, we obtained a previously unknown orthorhombic modification. This crystal modification was obtained for the CP complex with nickel under the same crystallization conditions as those used for the preparation of the trigonal modification.

Both crystal modifications of CP are characterized by high water content (70.0 and 67.3% for the trigonal and orthorhombic forms, respectively). This is one of

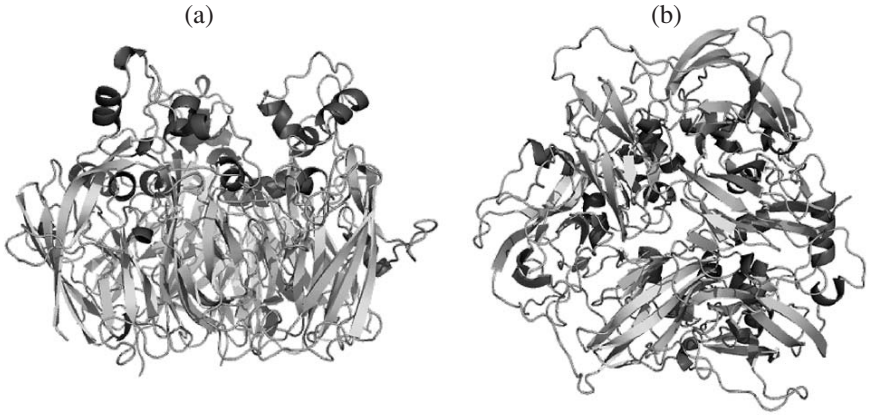

Fig. 2. Secondary structure of ceruloplasmin projected (a) along the threefold axis and (b) perpendicular to the threefold axis.

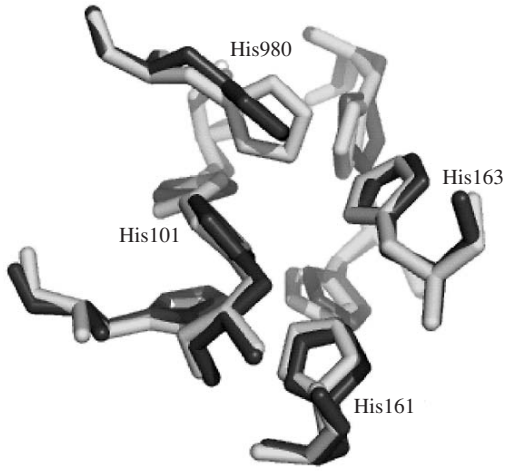

Fig. 3. Position of the residue His980 in the CP structures at 2.95 (light gray) and 2.6 Å resolution (dark gray).

the factors responsible for a rather low resolution at which the X-ray diffraction data were collected for the crystals of CP. Conceivably, the presence of long flexible loops on the molecular surface hinders the formation of a more close-packed crystal structure as well. As a consequence of the presence of these loops, there are rather wide channels in the crystal structure.

The trigonal structure contains channels with a virtually triangular cross section (Fig. 4a) running along the threefold axes, which are located in the centers of the channels. The size of the cross section is approximately ~110 × 110 × 110 Å. The contacts between the molecules are formed by amino-acid residues from four regions (Table 3). The largest region, 697–716, includes the residue R701, which is linked to the residues P829 and E834 of the adjacent molecule by hydrogen bonds (Fig. 5). The method used for the protein isolation in the earlier studies did not ensure complete inhibition of impurity proteinases, and CP was not, apparently, subjected to proteolysis at R701 in the crystals owing to the involvement of the loop 697–716 containing the latter residue in intermolecular contacts formed upon protein crystallization.

The orthorhombic and trigonal structures have different intermolecular-contact regions. The molecules are packed so that two types of channels (large and small) with diameters of about 43.3 and 90.0 Å, respectively, are formed along the short axis (Fig. 5b). The amino-acid residues involved in contacts are located in domains 1, 2, 4, 5, and 6 (Table 3). In this packing, the R701-containing loop points toward the center of the large channel and, consequently, is not involved in intermolecular contacts. The fact that the electron density for the region containing this residue is well defined can be attributed to efficient proteinase inhibition.

Labile metal-binding sites. As was shown earlier, CP contains not only three mononuclear and one trinuclear copper-binding site but also two so-called labile metal-binding sites in domains 4 and 6 [9–11]. In the site belonging to domain 4, the residues H602, E597, D684, and E971 (from domain 6) are coordinated as ligands to the metal atoms. In the site belonging to domain 6, the residues H940, E935, D1025, and E272

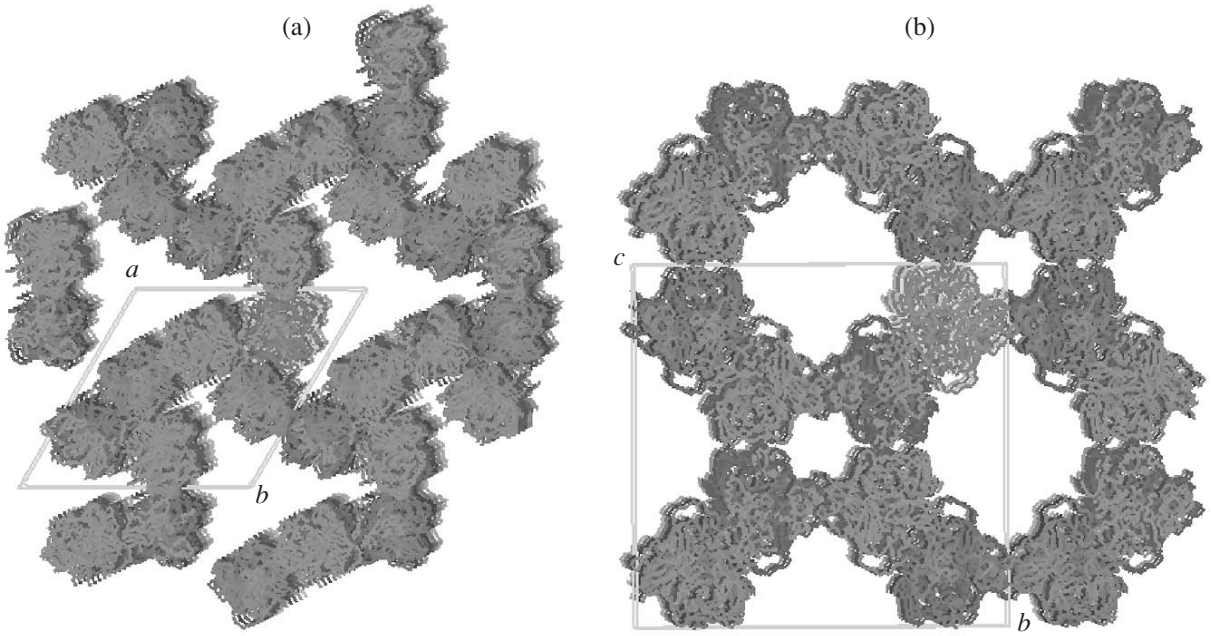

Fig. 4. Crystal packings of CP: (a) trigonal space group, (b) orthorhombic space group.

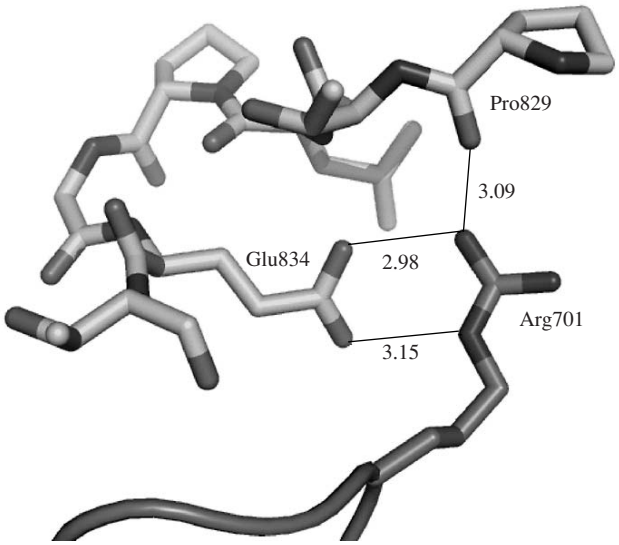

Fig. 5. Intermolecular contact involving R701 in the trigonal crystal structure.

(from domain 2) serve as ligands. The labile sites are located at a distance of around ~9 Å from the mononuclear sites of the corresponding domains. According to the results of the earlier studies, the labile sites are occupied predominantly by copper. The iron, nickel, zinc, and calcium contents are lower than 1, 1, 2, and 4%, respectively [17].

In the present study, the CP structure containing no ions in the labile sites was obtained for the first time. This is, apparently, associated with the use of EDTA for the protein isolation. This allowed us to reveal the conformational changes attendant upon ion binding in these sites.

In the CP structure with the unoccupied labile sites, the residues D684 (domain 4) and D1025 (domain 6) point outward, to the intermolecular-contact region, due to which the labile sites are accessible. According to the anomalous scattering data, the isolation of CP in the presence of a nickel salt led to the binding of Ni^{2+} in both labile sites (Fig. 6). The binding of the metal ions is accompanied by the twist of the carboxyl groups of D684 and D1025 (Fig. 7). The analysis of the anomalous difference electron density maps revealed two strong peaks with a height of 10σ. A comparison of the temperature factors shows that the site in domain 6 is completely occupied by nickel, whereas the occupancy of the site in domain 4 is 50%. In the structure of CP solved at 3.1 Å resolution [10], the labile sites are occupied by Cu^{2+} ions in a similar manner. In the structure solved at 2.8 Å resolution [11], only the labile site in domain 6 is half-occupied by copper, whereas the site in domain 4 is unoccupied. Conceivably, the copper ions were lost in the course of purification. Therefore, the copper ions in the labile sites are easily released and/or are replaced by other ions. Interestingly, the site in domain 4 is always less occupied than the site in domain 6, which may be indicative of the different functions of these sites.

Earlier investigations of the binding of various ions (Cu^{2+}, Co^{2+}, Fe^{2+}, and Fe^{3+}) in the labile sites [9] showed that the Cu^{2+} ions located in the labile sites of domains 4 and 6 are replaced by Co^{2+} ions. Copper ions in the labile sites can also be replaced by Fe^{2+} ions, but the latter migrate to putative binding sites (postulated by Lindley et al. [9]) after oxidation to Fe^{3+}. The Fe^{3+} ion replaces Cu^{2+} only in the labile site of domain 6 and also moves to the putative site.

Nickel is bound in the labile sites analogously to copper and cobalt ions. Since the concentration of nickel in blood plasma is normally very low compared to other microelements, the role of the labile sites of CP in the exchange of this metal is normally unlikely to be essential. However, the activity of CP decreases upon toxic poisoning with nickel or other heavy metals [33].

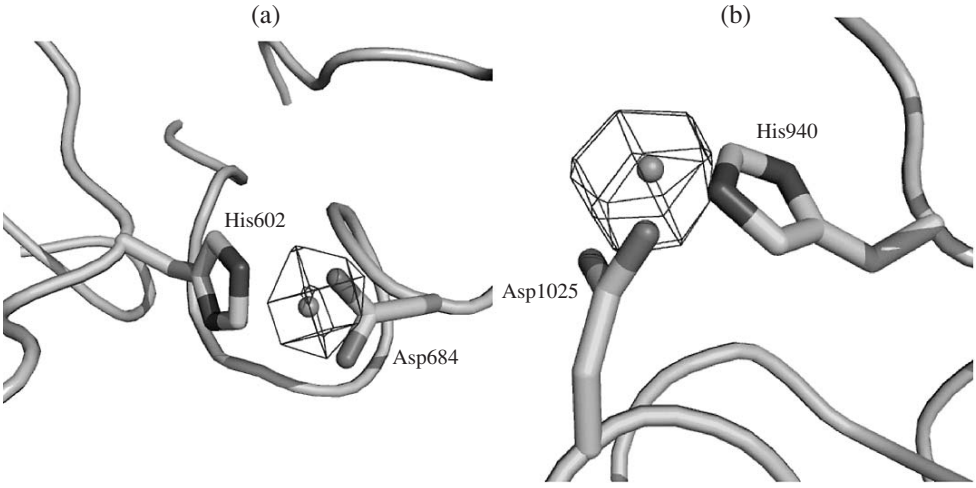

Fig. 6. Peaks in the anomalous difference electron density map in the Ni^{2+}-binding sites.

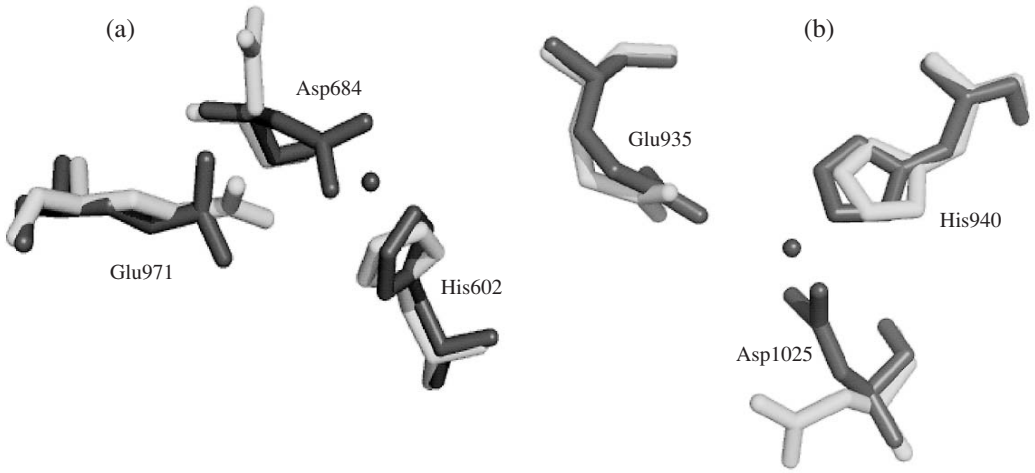

Fig. 7. Conformational changes observed upon metal binding in the labile sites of CP: (a) in domain 6; (b) in domain 4; CP-Ca^{2+} and CP-Ca^{2+} + Ni^{2+} are light gray and dark gray, respectively.

Chronic intoxication of rats with nickel causes a compensatory increase in the CP content [34]. It is conceivable that CP is involved in the multicomponent biochemical system of detoxication by binding heavy metals in the labile sites.

Another hypothesis about the role of the labile sites can be made on the basis of studies of the enzymatic properties of CP. In a study on the site-directed mutagenesis of the amino-acid residues that form the labile sites, the substitutions in both domains (E597A/H602A and E935A/H940A) were shown to cause a decrease in ferroxidase activity of CP [35]. Substitutions in domain 6 have no effect on oxidation of the synthetic diamine *o*-dianisidine. However, the substitution E971A in the labile site of domain 4 led to an increase in diamine oxidase activity. In our experiments, no substantial differences were found in oxidation of *o*-dianisidine catalyzed by CP containing or lacking Ni^{2+} ions. In CP, the binding site of biogenic amines (adrenaline, noradrenaline, serotonin, and dihydroxyphenylalanine) is located in domain 6 in the vicinity of the labile metal-binding site (E935, H940, D1025, and E272) and the mononuclear copper-binding site. The binding site of synthetic aromatic diamines is located in domain 4, also in the vicinity of the labile metal-binding site (E597, H602, D684, and E971) and the corresponding mononuclear site [10]. Hence it can be suggested, with a degree of certainty, that the electron transfer from the substrate to the copper ions in the catalytic site of CP rather than the binding of metal ions in the labile sites plays a decisive role in oxidation of *o*-dianisidine.

In addition to CP, a labile copper-binding site was found in copper-containing oxidase CueO at a distance

Table 3. Intermolecular contacts in CP (Å)

Trigonal sp. gr.			Orthorhombic sp. gr.		
molecule *A*	molecule *B*	distance	molecule *A*	molecule *B*	distance
Lys 557 NZ	Tyr 716 OH	3.04	Arg 774 NH2	Gln 985 OE1	2.50
Asn 697 ND2	Ser 771 OG	2.72	Thr 772 CG2	Glu 145 OE2	3.10
Arg 701 NH2	Pro 829 O	3.05	Thr 583 OG1	PHE 414 N	2.94
Arg 701 NH2	Glu 834 OE1	2.98	Arg 579 NH2	Thr 493 OG1	2.84
Arg 701 NE	Glu 834 OE2	3.15	Met 580 O	Lys 449 NZ	4.02
Glu 712 OE1	Asp 554 N	2.83	Arg 579 NH1	Pro 492 O	2.81
Lys 802 NZ	Asp 556 OD1	2.55	Ser 222 O	Asp 15 OD2	2.84
His 922 ND1	Ser 341 OG	3.55	Thr 219 O	Tyr 220 OH	3.13
Thr 837 N	Cys 699 O	3.16	Glu 223 OE1	Arg 46 NH2	3.23
Arg 125 NH1	Asp 122 OD1	2.83	Glu 223 OE2	His 20 N	2.49
Arg 125 NH2	Asp 122 OD2	2.85			

of 7.5 Å from the mononuclear site [36]. In spite of the fact that the labile binding site of CueO is not suitable for binding of Fe^{2+} ions, this oxidase exhibits weak ferroxidase activity in vitro. Like amino-acid substitutions in CP [35], amino-acid substitutions in the labile site of CueO lead to a decrease in oxidase activity of this enzyme [36]. On the contrary, copper ions in the unaltered labile sites cause an increase in oxidase activity of CueO [36]. The presence of iron ions leads to an increase in oxidase activity of CP [37]. Since these sites in each oxidase are suitable for labile binding of particular ions, the latter are conceivably involved in more efficient electron transfer to the type-I copper ion located in the catalytic sites of both CP and CueO. Apparently, the labile sites of CP are multifunctional. On the one hand, these sites can bind metal ions potentially toxic to organisms; on the other hand, they can facilitate electron transfer from iron ions in the ferroxidase reaction and can be involved in electron transfer from other substrates of CP. This is additional evidence that CP is a multifunctional protein playing a role in various processes proceeding in organisms.

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